



Biodiesel production using chemical and biological methods – A review of process, catalyst, acyl acceptor, source and process variables



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ABSTRACT

The indiscriminate extraction and consumption of fossil fuels have left the world with a corner kick into the area of exponential fuel demand and now the race is on for alternate energy source. The fortunate improvements in Biodiesel fuel production techniques has been the heading topic of economic and environment sustainability so far. Biodiesel have the potential to replace diesel in vehicle engines. It has been tested and proved that engines running on biodiesel have shown low smoke emission and low toxic gas emission. Biodiesel properties such as oxidation stability, cloud point, iodine value, linoleic acid and poly-unsaturated fatty acid methyl ester content of biodiesel are dependent upon the quality of the feedstock. Processing parameters such as density, viscosity, acid value, distillation property are dependent on feedstock as well as the reaction conditions or the extent of reaction. Combustion property greatly varies with the substrates used and almost all the varieties have been proven to be as superior as that of conventional diesel fuel. Though the existing fossil and terrestrial biomass based oil cannot realistically satisfy the existing demands, algal oil source scores the most out of demanded factors like oil content, extractability, comfortable cultivation and efficient biomass production. Algae are a diverse group of plant like microorganisms, prokaryotic and eukaryotic, mostly autotrophic in nature with basic requirement such as CO₂ and light for their normal growth and metabolic activity. Being micro scaled in physiology, most species of algae have less doubling time and the oil productivity greatly exceeds the outcome of best oil producing crops which clearly portrays that microalgae acts as a renewable source and can yield enough amount of oil for biodiesel production to meet the present intensifying demands. This article aims at reviewing the technical aspects of various biodiesel production methods from diverse oil feedstocks, their importance and significance of microalgal, process availability, commercialization potential of various processes.

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Contents

1. Introduction	369
1.1. Triglyceride as diesel fuel	370
1.2. Chemical composition	370
1.3. Properties of vegetable oil as fuel	370
1.4. Transesterification of fatty acids	370
2. Process of biodiesel production	370
2.1. Acid catalyzed process	370
2.2. Alkali catalyzed process	370
2.3. Super critical methanol process	371
2.4. Enzyme catalyzed transesterification – lipases	372
2.4.1. Extracellular lipase	373

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2.4.2.	Intracellular lipase or whole cell biocatalyst for biodiesel fuel production.	373
2.4.3.	Use of novel acyl acceptors in lipase catalyzed process.	374
3.	Process variables	374
3.1.	Selection of lipase/organism	374
3.2.	Substrates for biodiesel production	374
3.2.1.	Lipids	374
3.2.2.	Selection of acyl acceptors	374
3.2.3.	Selection of solvents	378
3.3.	Glycerol effect	378
4.	Production of biodiesel from microalgae	378
4.1.	Algae	378
4.2.	Algal diversity and global collection centers	378
4.3.	Biodiesel from microalgae	379
4.4.	Microalgae cultivation	379
4.4.1.	Open ponds (or) raceways	379
4.4.2.	Tubular photobioreactors	379
4.5.	Triglyceride synthesis	379
4.6.	Extraction and analysis of oil and biomass	379
4.7.	Significance of algal biofuel	380
5.	Conclusion	380
	References	380

1. Introduction

Alternate fuels for diesel engines are becoming increasingly important due to diminishing fossil reserves and studies on renewable resource have gained much importance in the present scenario. As it is known depleting petroleum resources can hinder our lifestyle, automobile industry will take a major blow and many other industry which are dependent on the fuels for energy will have to be closed. This situation will have a negative impact on the growth of the developing countries and also affect the developed countries. A realistic approach to the above problems will be to use an alternative fuel that is a green fuel with zero sulfur content and is obtained from renewable sources. Biodiesel production have gained reputed value in the catalog of renewable resource and known for its reduced toxic emissions when used in conventional engines after blending with Petro Diesel. Initially in 1980, scientists considered the direct usage of oils obtained from plants or animals as a potential replacement for diesel in automobiles directly [1] or by mixing or blending them with diesel. This did not prove fruitful as long term use in direct-injection engines caused several problems such as (i) Coking and trumpet formation on the injectors to such an extent that fuel atomization does not occur properly or is even prevented as a result of plugged orifices (ii) Carbon deposits (iii) Oil ring sticking and (iv) Thickening and gelling of the lubricating oil as a result of contamination by the vegetable oils. The reason for the above problems was identified as higher viscosity, lower volatility and the reactivity of unsaturated hydrocarbon chains present in the vegetable oils.

Beginning 1990, scientists found ways to reduce the viscosity and molecular weight of the vegetable oils by several ways. Notable among them was by pyrolysis and transesterification. Pyrolysis has disadvantages of being expensive and yield of undesirable products [2]. Hence research was extensively made on the transesterification and resulting fuel was called as biodiesel. Transesterification or alcoholysis of vegetable oil is a process by which fat or oil (i.e., glyceride) is made to react with an alcohol in the presence of catalyst to form an ester and glycerol. This ester is called as biodiesel. The commonly used alcohol is methanol and in some cases ethanol. Apart from the materials mentioned above an optional organic solvent is required for increasing the mutual solubility of the reactants [3]. Biodiesel is chemically known as Fatty Acid Alky Esters (FAAE) and the alkyl group is decided by the

acyl acceptor used for the reaction. FAAE has a molecular weight that is one-third of that of the vegetable oil, viscosity of about one-eighth and contains 10–11% oxygen (w/w) which enhances its combustion process. The cetane number of FAAE is better than vegetable oils and also the calorific value of FAAE [4]. The versatility of the biodiesel production lies in the various methods by which it can be produced commercially. It can be done by varying any one of the following: (i) Oil/Fat source (ii) Catalyst (iii) Acyl acceptor and (iv) Solvent. Previously scientists have reported carrying out transesterification processes using chemical catalysts like acid [5] and alkali [6] which gave high conversion rates but had a disadvantage of toxic waste accumulation. Then use of biochemical catalyst – an enzyme known as lipase was carried out. Lipase catalyzed transesterification gave good conversion and immobilization of the enzyme or the enzyme producing organism was analyzed which proved the reusability of the catalyst and removed the problem of toxic wastage. Usage of novel acyl acceptors like Dimethyl Carbonate, Methyl Acetate [7] or Ethyl Acetate increased the conversion rate and decreased the enzyme deactivation which was prevalent when methanol was used as the acyl acceptor. Furthermore using novel solvents like t-butanol increased the stability and yield of the process. Earlier only animal fat or edible and non-edible vegetable oils or waste cooking oil were used for biodiesel production. Usage of these oils has a disadvantage of limited availability and requirement of large area for the cultivation. Hence an alternative use of algae as oil source is advised. Algae are plant like prokaryotic or eukaryotic, single cellular or multicellular organisms. They are photosynthetic and

Table 1
General source of lipase [31,32].

Bacteria	Fungus	Plants
<i>Streptomyces</i> sp.	<i>Mucor</i> sp.	<i>Lycopersicon esculentum</i>
<i>Achromobacter</i> sp.	<i>Rhizopus</i> sp.	<i>Carissa carandas</i> fruit
<i>Alcaligenes</i> sp.	<i>Candida</i> sp.	Castor bean
<i>Arthrobacter</i> sp.	<i>Geotrichum</i> sp.	<i>Cucumis melo</i>
<i>Pseudomonas</i> sp.	<i>Aspergillus</i> sp.	<i>Hibiscus cannabinus</i>
<i>Bacillus</i> sp.	<i>Penicillium</i> sp.	<i>Lycopersicon esculentum</i>
<i>Staphylococcus</i> sp.	<i>Humicola</i> sp.	<i>Moringa olifera</i>
<i>Chromobacterium</i> sp.		Rice bran
<i>Burkholderis</i> sp.		<i>Triticum aestivum</i>

follow autotrophic or heterotrophic mode of nutrition. Algae are known for their high oil content and quick lipid productivity with less doubling time [8] by which most of the algal species are found to have the ability to be used as sustainable oil producing biomass.

1.1. Triglyceride as diesel fuel

Triglycerides from vegetable oils, animal oils are the renewable sources of energy for Biodiesel production. However, animal oil cannot meet the existing demands, fresh plant oil, waste cooking oil and microalgal oil are the expected alternate triglyceride sources that has the potential to meet the existing and future fuel demands of human kind. The use of vegetable oil as alternative renewable fuel was proposed in the beginning of 1980s [4] and now the process is in industrial practice

1.2. Chemical composition

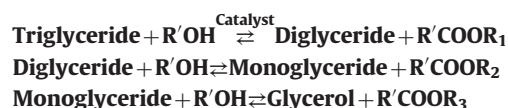
Glycerides are composed of hydrocarbon chains. They are rich in carbon and hydrogen but comparatively lacks in oxygen. Lipids produced by organisms are classified according to their chemical composition and their solubility is limited in water.

1.3. Properties of vegetable oil as fuel

Vegetable oil has been found up to the mark satisfying the property requirements as a renewable oil resource. Reasons for opting Triglyceride as alternate fuel source are discussed [9]. The significant factors are liquid nature-portability, ready availability, renewability, higher heat content, lower sulfur content, lower aromatic content, biodegradability etc. Disadvantages of using Triglycerides as source are high degree of viscosity, low volatility reactivity of unsaturated hydrocarbon chains.

1.4. Transesterification of fatty acids

Transesterification of triglycerides has been in application since the last decade. Fatty acids react with acyl acceptors to give Fatty acid alkyl esters. The alkyl group of resultant FFAE and the by-product depends on the acyl acceptor used. Glycerol is obtained as by-product in most cases where as use of dimethyl carbonate, ethyl acetate, methyl acetate results in various other by-products as discussed in latter sections. General equation for three step formulated Transesterification of Fatty acid is given below



2. Process of biodiesel production

Biodiesel production follows Transesterification principle. Transesterification (also called as alcoholysis) is the reaction of fat or oil with an alcohol to form esters and glycerol. Two step catalyzed production was also reported [10]. Usually a catalyst is used to mediate the reaction and bring out quicker reaction rate. After transesterification of triglycerides, the products are a mixture of esters, glycerol, alcohol, catalyst and tri-, di- and monoglycerides. Based on the process requirements, there are various methods that can be adopted for producing biodiesel from various oil sources. Some of them are described in a detailed fashion and follows as given below.

2.1. Acid catalyzed process

Acid catalysis is the most suitable method in case of organic substrates. The transesterification process is catalyzed by acids and these catalysts give very high yields of alkyl esters, but the reactions are very slow. The homogeneous acid catalysts are H_2SO_4 , HCl , BF_3 , H_3PO_4 and some organic sulfonic acids. Sulfonic and sulfuric acids are mostly preferred. At the most, 99% conversion rates have been reported by using acid catalysts [11]. Freedman and Pryde [12] got the desirable product with 1 mol% sulfuric acid with a molar ratio of 30:1 at 65 °C with 99% conversion rate in 51 h. When excess of acid is added, better conversion of triglyceride is obtained. Advantages of acid catalyzed transesterification are direct biodiesel production from low cost lipid feed stocks, such as waste cooking oil, greases etc. [13]. These oil sources have FFAs level of > 6% [14]. Though increased FFA content affects the conversion rates in acid catalyzed process, economy analysis depicts that Single-step acid mediated conversion is better than two-step alkali mediated conversion where FFAs requires additional one step to be converted to methyl esters [15]. To overcome the fatty acid related problems, since the system is liquid based, liquid acid catalysts are proposed. Acid addition leads to protonation of the carbonyl group of the ester that results in carbocation which, after a nucleophilic attack of the alcohol, produces the tetrahedral intermediate, which eliminates glycerol to form the new ester and regenerates the catalyst H^+ . According to the mechanism, carbocation can be allowed to undergo in presence of water, but Acid catalyzed transesterification of triglycerides are efficient in water absent systems [12,16]. Solid heterogeneous acid catalysts have the potential to replace liquid acid catalysts. Solid catalysts are insensitive to FFA content of the oil thus eliminating the need for regular removal of biodiesel and by-product from the reactors and enabling easy recovery and reuse of solid catalyst which in turn reduces corrosion problems. The solid catalyst is expected to have properties like interconnected system of large pores, enabled to tolerate high concentrations of acids and hydrophobic surface to promote the yield of ester conversions [13]. But unfortunately, problems rise with the glycerol recovery. This necessitates the experimental determination of optimal relations between the alcohol and raw materials [11].

2.2. Alkali catalyzed process

Most widely used homogeneous base catalysts are NaOH , CH_3ONa and KOH . Generally for homogeneous base catalysis either

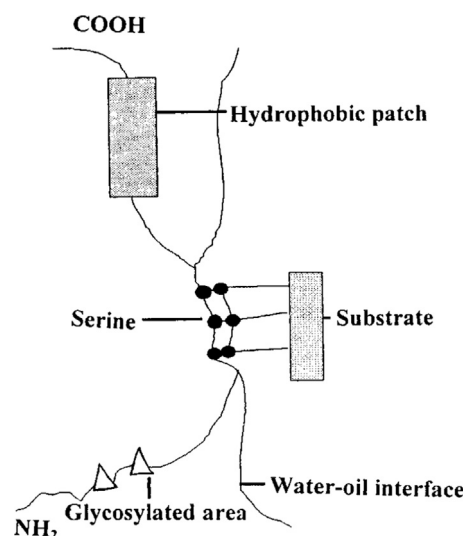


Fig. 1. Diagrammatic representation of a lipase molecule showing its main features [34]. Substrate is triglyceride.

sodium hydroxide (NaOH) or Potassium hydroxide (KOH) is used with methanol or ethanol as well as any kind of oils, refine, crude or frying. Alkaline metal oxide (CH_3ONa) are the most active

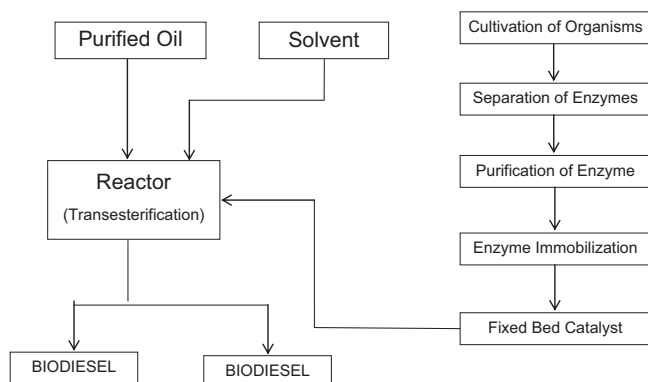


Fig. 2. Process flow sheet of biodiesel production by extracellular lipase.

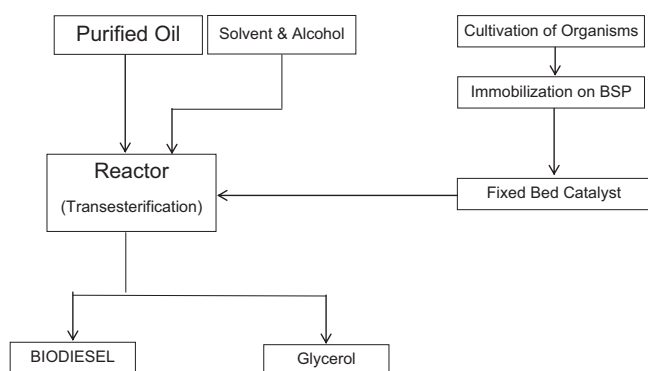


Fig. 3. Process flow for biodiesel production using intracellular lipase.

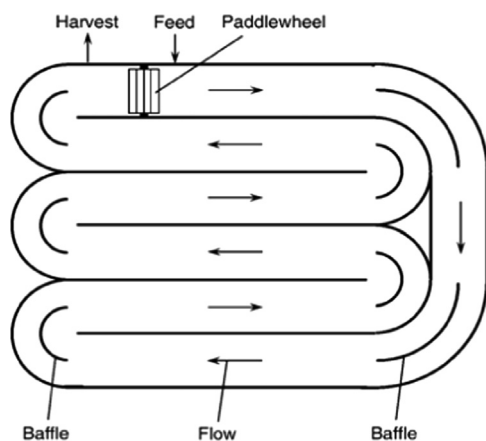


Fig. 4. Open pond system or raceways [8].

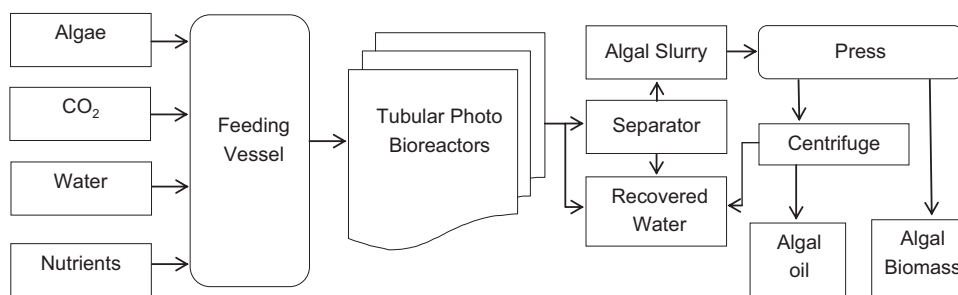


Fig. 5. Cultivation of algal biomass using Tubular Photo Bioreactors.

catalysts, since they have very high conversion rates ($> 98\%$) in short time (30 min) even when applied at lower concentration (0.5 mol%). Effect of moisture has negative impact in case of alkali catalyzed process because of soap formation that consumes the catalyst and reduces the efficiency, as well as causing an increase in viscosity. To obtain maximum conversion rates without the use of excess alcohol, molar ratio of 6:1 is preferred [11]. The type of alcohol is usually ethanol and methanol. The first one has fewer safety problems because it is less toxic and comparatively easy to handle. Reactions with alkali catalysts are found to perform quick than the acid catalyzed reactions [12,17,18]. Generally, alkali mediated reactions is a three step formulated process. The first step is the reaction of base with the alcohol, producing an alkoxide and the protonated catalyst. The nucleophilic attack of the alkoxide at the carbonyl group of the triglyceride generates a tetrahedral intermediate, from which the alkyl ester and the corresponding anion of the diglyceride are formed. The latter deprotonates the catalyst can react with second molecule of alcohol and start another catalytic cycle [4]. Standard value of the reaction to take place is 60°C , but depending on the oil source and catalyst, different degrees of conversion is obtained at various temperature ranging from 25 to 120°C [19–26]. Heterogeneous solid alkali catalysts are basic zeolites, alkaline earth metal oxides and hydrotalcites. They are easy to use, regenerate, supports recycling and to perform economically safer reactions. These catalysts can be directly used if the FFA content is less than or equal to 1. If, the amount of fatty acid is too big in case of waste cooking oil, then pretreatment via transesterification with alcohol but with sulfuric acid is recommended before initializing with alkali mediated conversion [11].

2.3. Super critical methanol process

A fluid is considered supercritical when its temperature and pressure go above its critical point. Supercritical Fluid (SCFs) possesses unique transport properties. They can effuse through solids like gas and dissolve materials like liquid. The above discussed simple transesterification process is confronted with two problems, i.e., the process is relatively time consuming and it needs separation of the catalyst and saponified impurities from biodiesel. Apart from these problems, reduced efficiency of catalyst and high catalyst consumption are other problems that formed the root cause for the design of supercritical methanol process. These problems are eliminated in the non-catalytic supercritical methanol method of transesterification. This is perhaps due to the fact that the tendency of two phase formation of oil/methanol mixture is not encountered and due to decrease in dielectric constant, single phase is encountered in the supercritical state of ethanol. Further, since the reaction is catalyst free, purification of biodiesel is easy, environment friendly and completes in 2–4 min [27]. This novel method was reported by Saka and Kusdiana [28] who demonstrated that preheating methanol at 350°C for 24 h was enough to convert rape seed oil to methyl esters. Molar ratio of

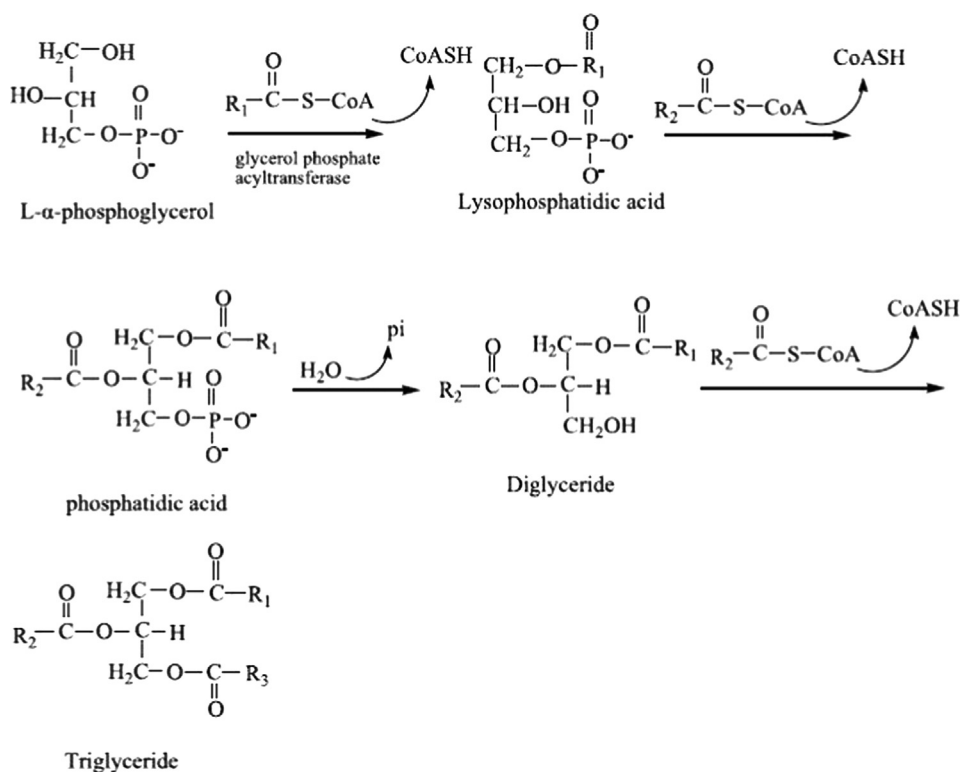


Fig. 6. Synthesis of triglycerides in microalgae.

Table 2

Some commercially available lipase producing microbial strains [34].

Type	Source	Application
Bacteria	<i>Psuedomonas fluorescens</i>	Organic synthesis
	<i>Burkholderia cepacia</i>	Organic synthesis
	<i>Chromobacterium viscosum</i>	Organic synthesis
	<i>Pseudomonas aeruginosa</i>	Organic synthesis
	<i>Burkholderia glumae</i>	Organic synthesis
Fungi	<i>Candida rugosa</i>	Organic synthesis
	<i>Candida antarctica</i>	Organic synthesis

Table 3

Yield of biodiesel using lipase from various sources using solvent free system.^a

Oil	Source of lipase	Alcohol	Reaction time (h)	Yield (%)
Tallow	<i>Candida antarctica</i>	Isopropanol	16	90.3
		2-Butanol	16	96.4
	<i>Mucor meihei</i>	Ethanol	5	65.5
		Iso-butanol	5	97.5
		Methanol	5	19.5
Palm oil	<i>Pseudomonas cepacia</i>	Methanol	8	15.0
		Ethanol	8	72.0
Coconut oil	<i>Pseudomonas cepacia</i>	1-butanol	8	40.0
Sunflower oil	<i>Pseudomonas fluorescens</i>	Methanol	24	3.0
		Ethanol	24	8.0
Cotton oil	<i>Candida antarctica</i>	Methanol	7	91.5

^a Based on data provided by Mirolawa et al. [114].

methanol to rapeseed oil of 42:1 was considered as the best ratio [29]. Effect of water is an important factor in conventional catalytic conversion of vegetable oils and mostly resulted in negative results with the presence. Since, super critical methanol have hydrophobic nature with a lower dielectric constant, non-polar triglycerides can be well solvated with supercritical methanol to

form a single phase oil/methanol mixture. However, liquid methanol is a polar solvent and has hydrogen bonding between OH oxygen and OH hydrogen to form methanol clusters [30]. Thus, yield of methyl esters is high in supercritical methanol process than any other conventional techniques. Yield of methyl esters increases with increase in molar ratio of oil to methanol. Due to severe reaction conditions and high operational costs, this method suffers few disadvantages and hence it is not a viable option for industry level commercialization. The uses of co-solvents such as carbon dioxide, hexane, propane, etc. are being investigated to reduce the operational parameters and make the process economical (Figs. 1–6).

2.4. Enzyme catalyzed transesterification – lipases

Alkali catalysis mechanism is widely applied to scale up the production in industries. Though alkali catalyzed processes are economic and efficient enough, lipase dependent catalysis have certain advantages over conventional catalysis. Lipase is the most versatile industrial enzyme and known to carry out range of bioconversions (Fig. 1). Lipase mediated reaction offers easy removal of glycerol by-product which is a necessary process to be carried out throughout the reaction to maintain the equilibrium in the positive domain (alkyl ester formation) in case of chemical mediated catalysis. Also, difficult glycerol recovery, removal of alkaline catalyst, energy intensiveness, treatment of highly alkaline waste water remains as a great challenge for industries. This serves as a main reason for replacing alkali catalyst with Biocatalyst. Lipase mediated reactions are environmentally friendly and do not promote side reactions. Extracted Lipase and some Lipase producing Microorganisms (mostly Fungi) are immobilized in biomass support particles and used as catalytic beds to obtain prolong use. It has been revealed that contribution of bacterial Lipase is 45%, fungal 21%, animal 18%, plant 11%, and algae 3% [31]. Some of the Lipase source (Microorganisms and plants) are listed in Table 1. Only some species of organisms are selected based on

few considerations such as activity of lipase, availability of species and amount of lipase produced to ensure the cost effectiveness and process efficiency. Microorganisms produce lipase in large quantities and hence whole organisms are chosen to act as immobilized catalytic beds. Commercial names of lipases are Novozym, Lipolase, Lipozyme, Lipomax, Lumafast [33] etc. Some commercially available Lipase producing Microbial Strains are given in Table 2.

2.4.1. Extracellular lipase

Extracellular lipase permits the efficient catalysis in conversion of fatty acids to alkyl esters. Extracellular enzyme catalysis requires the need of downstream processing techniques for enzyme extraction followed by immobilization to ensure repeated use (Fig. 2). The extracted lipase from any suitable source (mostly microorganisms) is immobilized in a suitable support and used as catalytic beds. In the absence of organic solvent, lipase powders can be purchased and used for active catalysis in mediums with 4–30 wt% water without which the enzyme is nearly inactive [34]. For preparing lipase solution, lipase (0.5 g) was dissolved in 5.0 ml of water, stirred for 1 h, followed by centrifugation at 3500g for 10 min. Supernatant was used as enzyme solution after dilution with water [34].

2.4.1.1. Immobilization of lipase enzyme and optimization. Many techniques and different carriers have been employed for immobilization of lipases to produce biodiesel. Lipases have been successfully immobilized on both hydrophilic and hydrophobic materials such as porous kaolite particle, macroporous resin and functionalized nanoscale SiO₂ spheres [3,35,36]. Immobilization of different lipases on macroporous polypropylene beds have been studied extensively which revealed that the successful adsorption of lipases on hydrophobic materials depends on structural features of “typical” lipases [37]. The catalytic activity sharply increases when the substrate forms a separate phase at which lipases are adsorbed. This phenomenon of “interfacial activation” was firstly observed by Sarda and Desnuelle [38]. The greatest part of the lipase structure seems to have a recurrent structural domain—an amphipilic amino acid chain called “lid” or “flap” that buries the active site [39,40]. It is related with the affinity of lipases on hydrophobic surfaces [39,41]. Most lipases exist in two conformations, that is, an open and a closed conformations. The closed one dominates in water solution while open one exists with the presence of hydrophobic interface. In the closed conformation the flap exposes the hydrophilic side towards the water and hydrophobic side towards the catalytic side. In presence of a hydrophobic substrate (Triglyceride), the hydrophobic part of lipase (catalytic site) changes in conformation, opens and adsorbs the substrate in open configuration [42]. The covalent attachment of lipase on styrene-divinyl benzene-polyglutaraldehyde support has been found to be twice stable than lipase immobilized on to styrene-divinylbenzene beds by hydrophobic interactions. The immobilized enzyme was stable and supposed to have same catalytic activity even after 30 days of storage at 4 °C [43]. The optimal pH conditions for high lipase activity ranges from 5.0 to 9.0 and protein concentration from 1.14 to 11.4 mg. Enzyme loading can be calculated by Salis using the following equation [42].

$$L = \frac{A_i - A_r}{M_s}$$

where L is enzyme loading; A_i is initial enzyme activity; A_r is residual enzyme activity; M_s is the mass of support (g). Loading is expressed in L (U/g support). Dizge [43] calculated the lipase activity

as follows:

$$\text{Lipase activity} \left(\frac{\text{U}}{\text{g support}} \right) = \frac{\text{activity of immobilized lipase}}{\text{amount of lipase used}}.$$

2.4.2. Intracellular lipase or whole cell biocatalyst for biodiesel fuel production

The use of extracellular lipase have been extensively studied and found to be expensive due to the complicated processes in the downstream techniques of enzyme extraction from microbial cells. These studies have prompted the use of microorganisms such as bacteria, yeast and fungal species as whole cell biocatalyst (Fig. 3). Some of the species of lipase producing microorganisms having the ability to act as whole cell biocatalyst have been discussed. *Rhizopus oryzae* have been reported as efficient whole cell biocatalyst by [44]. Catalytic activity of *Mucor meihei* has been discussed in [45]. *Candida antartica*, *Candida rugosa* and *Candida cylindracea* have been noted significantly for the lipase productivity [46]. *Pseudomonas cepacia* and *Pseudomonas fluorescence* are found to have the capability to produce enough lipase and thus can be employed as catalyst in the process of Methanolysis of oils [47]. Whole cell catalytic activity of engineered *Escherichia coli* and yeast strain has been discussed in detail along with the secretory mechanisms [44]. Immobilized recombinant *Aspergillus oryzae* expressing heterologous lipase is also found to be efficient for enantio-selective transesterification [48].

2.4.2.1. Whole cell immobilization or bed preparation. Immobilized *R. oryzae* cells were the first whole cell biocatalysts used in the process of transesterification that provides 1,3-positional specificity lipase. Cells were cultivated under normal conditions and then immobilized within biomass support particles (BSP). In some cases immobilized culturing of cells has been carried out [42]. For cultured cell immobilization, Filamentous fungus such as *Rhizopus* sp., and *Candida* sp. are grown in basal medium. Li [49] cultivated *R. oryzae* in a culture medium containing 30 g soybean oil, 70 g peptone, 1.2 g NaNO₃, 1.2 g KH₂PO₄ and MgSO₄ · 7H₂O in 11 parts of tap water. Flasks (500 ml) containing 100 ml of basal medium was taken and spores are inoculated aseptically followed by incubation for 72 h at 35 °C on a Reciprocator shaker (130 oscillations/min) with 80 BSPs subjected to prior sterilization. The BSPs used were 5 mm cubes of reticulated polyurethane foam with a particle voidage exceeding 97% and a pore size of 50 pores per linear inch. Apart from *R. oryzae*, *Rhizopus chinensis* is also cultured and found to be active in non-aqueous system containing n-heptane [50]. For immobilized cell culturing, Hama [42] used Sakaguchi flasks (500 ml) containing 100 ml of basal media with glucose followed by sterilization and inoculated the spores of *R. oryzae* from a fresh agar slant aseptically. The inoculated flasks were incubated on a reciprocator shaker at 30 °C for 24 h (150 oscillations/min; amplitude 70 mm). The resultant culture broth was transferred to a 20-l air lift bioreactor, containing 101 basal media with 30 g/l of olive oil and 24,000 BSPs (6 mm × 6 mm × 3 mm cuboids). Aeration causes liquid and particle mixing and finally, temperature is maintained for 30 °C hours. After cultivation the BSP immobilized cells were separated from the culture and stabilized. These catalytic beds are more stable and efficient in packed bed reactors than bottle flasks. Lipase can be localized using immunofluorescent detection technique using rabbit anti-ROL IgG in phosphate buffer saline (PBS).

2.4.2.2. Pretreatment and stabilization. Efficiency of conversion rate in whole cell catalyzed systems also depends on the cell wall fatty acid composition of the immobilized organism [44]. Pretreatment

of catalytic beds triggers increased production of lipase by immobilized microorganisms. There are two types of lipases, one bound to cell wall (ROL 34) another bound to cell membrane (ROL 31). It is reported that the enzyme activity increases with addition of olive oil to catalytic beds before the reaction [51]. It has been reported that the increased catalytic activity is due to the increase in production of membrane bound lipase (ROL 31). Hence ROL 31 plays a major role in methanolysis activity. For pretreatment, substrate related compounds of lipase such as triglycerides and fatty acids acts as inducers in filamentous fungus [52]. Cells can also be pre-incubated in soy bean oil for some time before subjecting to methanolysis. For methyl ester pretreatment, oil is replaced with methyl ester and used. Whole cell immobilization permits prolonged use of catalytic beds thus catalyzing number of reaction cycles which depends on various other factors such as solvent used and volume considerations.

2.4.3. Use of novel acyl acceptors in lipase catalyzed process

Apart from the regular acyl acceptors like methanol and ethanol, researchers have also proposed the use of other novel acyl acceptors such as methyl acetate, ethyl acetate [14] and dimethyl carbonate [105]. Use of excess alcohol for the transesterification process gives rise to practical problems such as deactivation of the immobilized lipase specially by lower chain alcohols like ethanol and methanol [106,107]. Regular removal of by-product is advised to avoid process reversion since the process is reversible [105]. Some alternative ways such as use of solvents like hexane [108] or t-butanol [109] are also reported. The use of low concentration of methanol in ratio of 0.33:1 to oil was reported for the first problem and stepwise addition of alcohols was proposed for the second problem [106,112]. Even though, the alternatives showed good results, they increased the operational cost and complex process requires more man power.

2.4.3.1. Use of methyl acetate. Du et al. [110] suggested the use of methyl acetate instead of alcohol. High yield of 92% was obtained for the molar ratio of 1:12 of oil to methyl acetate. The yield was obtained for crude and refined soybean oil. The lipase B from *C. antarctica* was immobilized on acrylic resin and the enzymes remained active for 100 batches. The by-product obtained is triacetin glycerol and not glycerol and hence the loss of activity due to glycerol was eliminated.

2.4.3.2. Use of ethyl acetate. Use of ethyl acetate as an acyl acceptor for lipase catalyzed transesterification was reported [111]. Lipase B from *C. antarctica* immobilized on acrylic resin was allowed to react with crude oil of Jatropa, Karanj and Sunflower oil. A yield of 91.3%, 90% and 92.7% of ethyl ester was obtained respectively when reaction was processed with molar ratio of 11:1 of ethyl acetate to oil at 50 °C with reaction period of 12 h. The activity of enzyme remained enough efficient till 12 repeated cycles. The by-product obtained was triacetin, a valuable molecule which has wide spread application. The advantage of ethyl ester is that the extra carbon atom increases the heat content and cetane number. Ethyl esters also have lower pour and cloud points, higher flash and combustion points which improve cold starts and safety in handling. Smoke opacity and exhaust temperature is also lowered [14].

2.4.3.3. Use of dimethyl carbonate. Use of methyl acetate and ethyl acetate has a disadvantage that a large amount of compound is required (1:12 of oil/methyl acetate; 1:11 of oil/ethyl acetate). Hence another acyl acceptor, dimethyl carbonate was used [105]. DMC is a neutral, odorless, cheap, non corrosive, non-toxic compound that exhibits good solvent properties [113]. Advantage of DMC involved transesterification is the reaction remains in

positive product formation side (Biodiesel) as the product is CO₂ which escapes as gas.

3. Process variables

For efficient biodiesel production using enzyme catalyzed transesterification certain crucial parameters need to be addressed. The optimization of these parameters is significant in obtaining maximum yields. The basic parameters that are considered to affect the conversion rates are selection of lipase, selection of substrates, molar ratio of substrates, temperature, pH of lipase micro environment, water content, solvent content, glycerol content, mixing intensity and purity of reactants.

3.1. Selection of lipase/organism

Selection of the lipase mainly depends on whether the system is a solvent involved system or a solvent independent system, type of fatty acid involved and the lipase is to be used intra-cellular or extra-cellular along with other reaction parameters. An organic solvent such as n-hexane, s-butanol, petroleum ether are added to a system to increase the miscibility between the triglyceride and methanol and thus increasing the catalytic efficiency of lipase. Reactions can also be carried out in a solvent free system. Lipases from various microbes give various results in different systems and they are given in Table 3. The triacyl glycerol (TAG) and free fatty acids (FFA) present in the oil decides the activity of the lipase. Specificity of lipases for biodiesel synthesis refers to their region specificity or specificity with respect to the length of hydrocarbon chain of fatty acid [115]. The lipases selected for the catalysis are those that display wide substrate specificity. Examples are lipases from *pseudomonas* and *candida* species [116,117]. Lipases from other sources are also found to show considerable yields depending on the reaction parameters. Selection of the right lipase for the particular source and condition is necessary for higher yields. Lipase used for biodiesel production can be either used directly by immobilizing it in a suitable matrix or the whole organism can be used for the catalysis purpose. Whole cell biocatalysts are considered cheaper and hence are appropriate for industrial biodiesel manufacturing, but they are found to have a disadvantage of reduced conversion rates compared to immobilized lipases [121].

3.2. Substrates for biodiesel production

3.2.1. Lipids

Plant oils (refined and raw), oil from micro algae, waste fats which are remained after cooking are considered for biodiesel production. Depending on the geographical region and crops grown the oil for the biodiesel production are considered. Watanabe et al. [118] compared the effective transesterification of three form – raw, refined and degummed soya bean oil for using lipase from *C. antarctica*. The result showed lesser conversion in raw oil due to the presence of phospholipids. High amount of phospholipids gave low flame [110]. The phospholipid problem can be addressed by immersion pre-treatment of lipase (*C. antarctica*) in crude oil for 12 h, oil degumming [120] and simultaneous dewaxing/degumming was found to be efficient [110,120]. Even though refined oil gave higher yields, it was costly. Oil from micro algae is also potential source for biodiesel production. Hence proper selection of the source is important for industrial implementation (Tables 4 and 5).

3.2.2. Selection of acyl acceptors

Transesterification involves two substrates, one being the oil and other being an acyl acceptor. The acyl acceptor is crucial for

Table 4

Transesterification of oils from various sources.

Oil	Catalyst	Type	Catalyst amount (%)	Alcohol	Oil to alcohol molar ratio	Reaction conditions	Ester yield	Refs.
Rice bran oil	NaOCH ₃	Alkali, homogeneous	0.88	–	–	65 °C, 1 h	83.3	[53]
	–	Acid, homogeneous	–	Methanol	1:10	60 °C	< 96	[54]
Cotton seed oil	NaOCH ₃	Alkali, homogeneous	0.75	–	–	75 °C, 1.5 h	97	[55]
	TiO ₂ /SO ₄ ^{2–}	Acid, heterogeneous	2	–	1:12	230 °C, 8 h	90	[56]
	Lipase (<i>Candida antarctica</i>)	Enzyme	1.6	t-Butanol	1:4	50 °C, 24 h	95	[57]
	–	Super critical condition	–	Methanol	1:41	350 °C, 8 min	98	[58]
	–	Super critical condition	–	Ethanol	1:41	350 °C, 8 min	75	[58]
	–	Super critical condition	–	Methanol	1:41	250 °C, 8 min	85	[59]
Soybean oil	KOH	Alkali, homogeneous	0.5, 1, 1.5	Methanol	1:3, 1:6, 1:9	50–110 °C, 2–50 min	99	[60]
	Ca(OCH ₂ CH ₃) ₂	Alkali, heterogeneous	3	Ethanol	1:12	75 °C, 3 h	91.8	[61]
	Lithium-doped ZnO	Alkali, heterogeneous	–	Methanol	1:12	65 °C, 3 h	96.3	[62]
	Eu(NO ₃) ₃ /Al ₂ O ₃	Alkali, heterogeneous	–	Methanol	1:6	70 °C, 6 h	63	[62]
	KF/ZnO	Alkali, heterogeneous	–	Methanol	1:10	65 °C, 9 h	87	[62]
	H ₂ SO ₄	Acid, homogeneous	3	n-Butanol	1:1	120 °C, 1 h	–	[63]
	Trifluoroacetic acid	Acid, homogeneous	2	Methanol	1:20	120 °C, 5 h	98.4	[64]
	S-ZrO ₂ sulfated zirconia	Acid, heterogeneous	5	Methanol	1:20	120 °C, 1 h	98.6	[65]
	Al ₂ O ₃ /ZrO ₂ /WO ₃	Acid, heterogeneous	4	Methanol	1:40	250 °C, 20 h	90	[66]
	SBA-15-SO ₃ H-P123	Acid, heterogeneous	10	Methanol	1:20	75 °C, 20 h	85	[67]
	Silica-bonded N-propyl sulfamic acid	Acid, heterogeneous	7.5	Methanol	1:20	149.85 °C, 60 h	90.5	[68]
	Lipase (<i>Thermomyces lanuginosus</i>)	Enzyme	15	–	1:7.5	31.5 °C, 7 h	96	[69]
	Lipase (<i>Candida antarctica</i>)	Enzyme	3	Ethanol	1:2	50 °C, 1.5 h	83.5	[70]
	–	Supercritical Condition	–	Methanol	1:40	350 °C, 35 Mpa, 25 min	–	[71]
	–	Supercritical Condition	–	Ethanol	1:40	20 Mpa, 350 °C, 15 min	80	[72]
Sunflower oil	NaOH	Alkali, homogeneous	1	Methanol	1:6	60 °C, 2 h, 600 rpm	94	[73]
	KOH	Alkali, homogeneous	–	Methanol	1:6	25 °C,	90	[74]
	Magnesium-lanthanum mixed oxide	Alkali, heterogeneous	5	Methanol	1:53	65 °C, 30 min	100	[75]
	Lipase (<i>Pseudomonas fluorescens</i>)	Enzyme	10	Hexane	1:4.5	40 °C, 48 h	91	[99]
	Lipase (<i>Candida antarctica</i>)	Enzyme	30	Ethanol	1:5	45 °C, 12 h	27	[70]
	Lipase (<i>Mucor meihei</i>)	Enzyme	20	Ethanol	1:11	45 °C, 5 h	82	[70]
	–	Supercritical condition	–	Methanol	1:40	350 °C, 200 bar, 40 min	96	[76]
	–	Super critical condition	–	Ethanol	1:40	400 °C, 20 MPa, 40 min	100	[72]
Mahua	H ₂ SO ₄	Acid, homogeneous	0.32	–	1:24 (v/v)	60 °C, 1 h	98	[77]
Palm	NaOH	Alkali, homogeneous	1	–	–	70 °C, 30 min	95	[78]
	SO ₄ /SnO ₂	Acid, heterogeneous	3	Methanol	1:6	200 °C	80.6	[79]
	ZrO ₂ /SO ₄ ^{2–}	Acid, heterogeneous	1	–	1:6	200 °C, 1 h	90.3, 80.6	[80]
	KF/Al ₂ O ₃	Alkali, heterogeneous	4	Methanol	1:12	65 °C, 3 h	90	[81]
	CaO/Al ₂ O ₃	Alkali, heterogeneous	–	Methanol	–	65 °C, 3 h	< 95	[82]
	Lipase (<i>Pseudomonas fluorescens</i>)	Enzyme	20	Ethanol	1:18	40 °C, 24 h	98	[70]
	Lipase (<i>Candida antarctica</i>)	Enzyme	30	Ethanol	1:5	45 °C, 10 h	75	[70]
	–	Supercritical condition	–	Methanol	1:45	350 °C, 60 min	60.30	[83]
	–	Supercritical condition	–	Ethanol	1:50	400 °C, 20 MPa, 30 min	95	[72]

Table 4 (continued)

Oil	Catalyst	Type	Catalyst amount (%)	Alcohol	Oil to alcohol molar ratio	Reaction conditions	Ester yield	Refs.
Karanja oil	KOH	Alkali, homogeneous		Methanol tetrahydro-furan	1:10	60 °C	92	[84]
	KOH	Alkali, homogeneous	1	Methanol	1:6	65 °C, 2 h, 360 rpm	98	[85]
	Li/CaO	Alkali, heterogeneous	2	Methanol	1:12	65 °C, 8 h	94.8	[14]
Tobacco	H ₂ SO ₄	Acid, homogeneous	1	Methanol	1:18	60 °C, 25 min	91	[86]
Refined sunflower oil	CH ₃ ONa, CH ₃ OK	Alkali, homogeneous	1	Ethanol	1:3, 1:6, 1:9	30–80 °C, 3–60 min	46.3–99.6	[87]
Rubber seed oil	NaOH	Alkali, homogeneous	0.5	Methanol	1:9	45 °C, 30 min	–	[88]
Rapeseed oil	KOH	Alkali, homogeneous	1	Methanol	1:6	65 °C, 2 h, 600 rpm	96	[89]
	Styrene-divinyl benzene macroporous KF/Eu ₂ O ₃	Acid, heterogeneous	10	Methanol	1	100–140 °C, 4 h	16.5–55	[90]
		Alkali, heterogeneous	–	Methanol	1:12	Methanol reflux, temperature, 1 h	92.5	[62]
	Mg-AL HTs	Alkali, heterogeneous	–	Methanol	–	65 °C, 3 h	< 94	[82]
	Lipase (<i>Candida antartica</i>)	Enzyme	3	t-Butanol	1:4	35 °C, 12 h	95	[91]
	–	Supercritical condition	–	Methanol	1:24	250 °C, 6 MPa, 10 min	97	[92]
	–	Supercritical condition	–	Ethanol	1:42	350 °C, 25 MPa, 12 min	100	[72]
Waste cooking oil	NaOH	Alkali, homogeneous	1.1	Methanol	1:7	66 °C, 33 min	88.8	[93]
	KOH	Alkali, homogeneous	1	Methanol	1:6	70 °C, 1 h	98.2	[94]
	H ₂ SO ₄	Acid, homogeneous	41.8	Methanol	1:245	70 °C, 4 h	99	[95]
	Zeolite Y (Y 756)	Acid, heterogeneous	–	Methanol	1:6	460 °C, 37 min	26.6	[93]
	SO ₄ ^{2–} /SnO ₂ –SiO ₂	Acid, heterogeneous	3	Methanol	1:15	150 °C, 3 h	92.3	[93]
	TiO ₂ /MgO	Acid, heterogeneous	–	Methanol	1:30	150 °C, 6 h	92.3	[62]
	K ₃ PO ₄	Alkali, heterogeneous	4	Methanol	1:6	60 °C, 2 h	97.3	[93]
Jatropha oil	Lipase (<i>Candida antartica</i>)	Enzyme	4	–	1:3	30 °C, 50 h	90.4	[93]
	KOH	Alkali, homogeneous	1	–	–	75 °C, 1 h	97.6	[96]
	Alumina loaded with potassium nitrate	Alkali, homogeneous	6	Methanol	1:12	70 °C, 360 min	84	[97]
	NaOH	Alkali, homogeneous	1	Methanol	1:5	65 °C, 90 min	98	[97]
	KNO ₃ /Al ₂ O ₃	Acid, heterogeneous	6	Methanol	1:12	70 °C, 6 h	84	[98]
	Na doped SiO ₂	Alkali, heterogeneous	–	Methanol	1:15	65 °C, 45 h	99	[62]
	CaO	Alkali, heterogeneous	–	Methanol	–	70 °C, 2.5 h	< 93%	[82]
	Lipase (<i>Chromobacterium viscosum</i>)	Enzyme	10	–	1:4	40 °C, 10 h	92	[99]
	Lipase (<i>Candida antartica</i>)	Enzyme	30	Ethanol	1:5	45 °C, 10 h	55	[70]
	Lipase (<i>Psuedomonas cepacia</i>)	Enzyme	10	Ethanol	1:4	50 °C, 8 h	98	[70]
	–	Super critical condition	–	Methanol	1:40	350 °C, 200 bar, 40 min	> 90	[100]
	–	Super critical condition	–	Ethanol	1:50	400 °C, 20 MPa, 30 min	90	[72]
Neem oil	NaOH	Alkali, homogeneous	0.7	Methanol	1:6	60 °C, 6.5 h	94	[101]
Corn oil	p-Toluene sulfonic acid (PTSA), benzene sulfonic acid, H ₂ SO ₄	Acid, homogeneous	1–4	Methanol	10–3	40, 60, 80 °C, 20 min	90.2–97.1	[102]
Canola oil	Lewis acids (AlCl ₃ , ZnCl ₂)	Acid, homogeneous	1	Methanol	1:24	110 °C, 18 h	98	[103]
	H ₂ SO ₄ , benzene sulfonic acid, p-Toluene sulfonic acid or 2,4-dimethylbenzene sulfonic acid	Acid, homogeneous	1–5	Methanol	1:15	60 °C, 30 min	21–99.9	[104]
	Na, K, LieBaO	Alkali, heterogeneous	–	Methanol	–	50 °C, 4 h	< 97.5	[82]

Table 5
Oil yield from various biomasses.

S. no	Biomass	Oil content (% oil by wt in biomass)	Land use (m ² year/Kg biodiesel)	Oil yield (l/ha)	Biodiesel productivity (kg biodiesel/ha year)	Uses	Reference
1	Rubber seed	50	–	80–120	–	Nutrient rich feed for livestock, biodiesel production etc.	[137,141]
2	Hemp	33	31	363	321	Fiber, feed for livestock, biodiesel production etc.	[137,141]
3	Corn/maize	44	66	172	152	Food, feed for livestock, biodiesel production etc.	
4	Soybean	18	18	446	562	Protein rich supplements, biodiesel production etc.	[137,141]
5	Safflower	–	–	779	–	Flavoring foods, nutritional supplements, biodiesel production etc.	[137,141]
6	Chinese tallow	–	–	907	–	Ornamental tree and oil for biodiesel production.	[137,141]
7	Camelina	42	12	915	809	Omega-3-supplement, edible oil and Biodiesel production.	[137,141]
9	Rice bran	16–32	–	–	–	Non-edible vegetable oil, replacement of mineral diesel etc.	[13]
8	Sunflower	40	11	952	946	Edible oil, biodiesel production etc.	
9	Nahor	58–75	–	–	–	Wood for railway crossties, boat building, mine props, biodiesel fuel etc.	[13]
9	Peanut	–	–	1059	–	Lactose free milk like beverage, oil for biodiesel production.	[137,141]
10	Canola/rapeseed	41	12	1190	862	Edible oil, biodiesel production etc.	
12	Moringa	33–41	–	–	–	Medicinal use, leaves as greens in food, oil for biodiesel etc.	[13]
12	Castor	48	9	1413	1156	Adhesives, coatings, soaps, lubricants, paints and dyes etc.	[13]
13	Jatropha	28	15	1892	656	Biodiesel, mineral diesel substitute, live fence, to reclaim land and control erosion.	[13]
14	Karanj	–	–	2590	–	Tanning leather, soap, lubricant, water-paint binder, pesticide etc.	[140]
15	Coconut	–	–	2689	–	Edible oil, kernel, biodiesel production etc.	[140]
16	Mahua	35–40	–	–	–	Ointment, rheumatism, lubrication, illumination, soaps, etc.	[13]
17	Linseed	35–45	–	–	–	Geotextiles, oil crop, stem fibers, paper manufacturing etc.	[13]
18	Oil palm	36	2	5950	4747	Cosmetic, soaps, lubricants, transportation fuel and substitute for biodiesel.	[137,141]
17	Microalgae (low oil content)	30	0.2	58,700	51,927	Human nutrition, Animal feed, aquaculture, bio-fertilizers, recombinant proteins, source of poly unsaturated fatty acids, methane production etc.	[8,13,135–137]
18	Microalgae (medium oil content)	50	0.1	97,800	86,515		[8,13,135–137]
19	Microalgae (high oil content)	70	0.1	136,900	121,104		[8,13,135–137]

Table 6
Oil content of few open pond microalgae species [8].

Microalga	Oil content (% dry weight)
<i>Botryococcus braunii</i>	25–75
<i>Chlorella</i> sp.	28–32
<i>Cryptocodinium cohnii</i>	20
<i>Cylindrotheca</i> sp.	16–37
<i>Nitzschia</i> sp.	45–47
<i>Phaeodactylum tricornutum</i>	20–30
<i>Schizochytrium</i> sp.	50–77

Table 7
Typical characteristics of fossil oil and bio-oil from microalgae [136].

Properties	Unit	Standard	Bio-oil from microalgae	Fossil oil
Density	Kg/l	ASTM D-6751	1.16	0.75–1
Viscosity	Pa/s	ASTM D-445	0.1 @ 40 °C	2–1000
Heating value	MJ kg ⁻¹	ASTM D-6751	29	42
Stability	–	ASTM D-6751	Not as stable as fossil fuels	–

the production of the bio diesel. Several acyl acceptors currently under study are alcohols such as methanol, ethanol, iso propanol, iso butanol, 2-butanol and 1-butanol or other novel acyl acceptors such as methyl acetate, ethyl acetate and di methyl carbonate. Methanol and ethanol are industrially produced in large scale and are cheap. Use of ethanol as acyl acceptor gives bio diesel with an additional carbon atom which increases the fuel properties. One solution to denaturation by methanol is stepwise addition of the

alcohol [121]. By this process 1/3 M equivalent of methanol was added for the stoichiometric amount of the waste oil and the eluate of the first step is again treated with 1/3 M equivalent of methanol. Immobilized *C. antartica* was taken and the yield was reported more than 90%. Nelson et al. [122] reported that compared yields of transesterification of methanol, ethanol and isobutanol with follow in presence of immobilized lipozyme IM 60 (M. Michel) and novozyme SP435 (*C. antartica*) the yield was 83.8% for isobutanol in hexane solvent and in solvent free system the yield for isobutanol was highest with 97.4%. This shows that isobutanol can also be used as an acyl acceptor for lipase catalyzed transesterification. The draw back with alcohols is that they inactivate the lipase at higher concentration. It has been noted that the degree of deactivation has been found to be inversely proportional to the number of carbon atoms in the linear lower alcohols [107]. The simultaneous presence of water with methanol can speed up denaturation of lipase while in systems containing ethanol, propanol, isopropanol, butanol the presence of small amounts of water is necessary [119]. Methyl acetate was used as an acyl acceptor by Du et al. [119] and a yield of 92% was reported for rude soya bean oil in the presence of lipase from *C. antartica*. Prasad et al. used ethyl acetate and the yield for jatropha, karanj and sunflower oils were 91.3%, 90% and 92.7% lipase from *C. antartica* was used. The acyl acceptor used in both the process involve high amount of lipase (three fold more) and molar ratio of oil to acyl acceptor is 1:12 and 1:11 respectively. Use of dimethyl carbonate by Wei et al. [105] gave a yield of > 90% for cotton seed oil, olive oil, sunflower oil, corn seed oil, rapeseed oil and soybean oil in presence of organic solvent petroleum ether. Use of DMC gives an irreversible reaction.

Table 8

Comparison of properties of microalgal oil, conventional diesel fuel and ASTM biodiesel standard [152].

Properties	Biodiesel from microalgal oil	Diesel fuel	ASTM biodiesel standard
Density (kg l^{-1})	0.864	0.838	0.84–0.90
Viscosity ($\text{mm}^2 \text{s}^{-1}$, cSt at 40 °C)	5.2	1.9–4.1	3.5–5.0
Flash point (°C)	115	75	Min 100
Solidifying point (°C)	–12	–50 to 10	–
Cold filter plugging point (°C)	–11	–3.0 (max –6.7)	Summer max 0
Acid value (mg KOH g^{-1})	0.374	Max 0.5	Winter max < –15
Heating value (MJ kg^{-1})	41	40–45	–
H/C ratio	1.81	1.81	–

3.2.3. Selection of solvents

Solvents are used in biodiesel production in order to increase the mutual solubility of hydrophobic tri acyl glycerides and hydrophobic alcohols. Poor solubility of short chain alcohols such as methanol and ethanol causes inhibition on the enzyme activity. It has been observed by Laane et al. [123] that the log *P* value was the fundamental property that described the influence of polarity-hydrophobicity of organic solvents on enzymatic catalysis. An ideal solution must ensure good solubility of oil and alcohol along with maintaining the enzyme stability. Use of a mixture of solvents can be done [124] in order to get better results. Among many organic solvents present certain organic solvents were observed to give higher yields and provide more stability to the enzyme. They are hydrophobic ones such as isooctane (log *P*=4.7), n-heptane (log *P*=4.0), petroleum ether 60–120 (log *P*=3.5–4.3), petroleum ether 20–40 (log *P*=3.2), n-hexane (log *P*=3.5), cyclohexane (log *P*=3.1) tert-butanol (log *P*=0.83). Hydrophobic ones such as acetone (log *P*=–0.24), n-hexane (log *P*=–3.5), diesel oil (log *P*=–8.0). Although there are many reasons for using an organic solvent in biodiesel production by transesterification, organic solvents also have certain draw backs such as they being volatile, flammable and toxic. These negative attributes make them a bane for economic biodiesel production. Also organic solvents make the biodiesel recovery difficult during down steam operations and certain organic solvents (hydrophobic solvents like n-hexane and petroleum ether) do not completely solubilize methanol and glycerol [125]. Hence their purpose is not completed. To address these issues several interesting ways have been proposed. One being using tert-butanol as solvent which is a moderate polar solvent which solubilises both methanol and glycerol [125], second being the use of diesel as organic solvent [126] which will eliminate the processes involved in solvent recovery and the third being the use of solvent free system where in the methanol is added in batches [112].

3.3. Glycerol effect

In continuous and repeated batch process glycerol was formed to influence the transesterification process by inactivating the enzyme. Dossat et al. [127] and Du et al. [119] observed a drop in activity of lipases by glycerol when the former was adsorbed in a hydrophilic matrix. This was due to the adsorbing of glycerol molecules onto the surface of the matrix which resulted in the formation of hydrophilic coating of glycerol which made the enzyme molecules inaccessible to hydrophobic substrates (reduced diffusion). Another possible mechanism was proposed for inhibition of glycerol was that glycerol may cause a decrease in the water activity of the enzyme. Several novel methods were

proposed to counter the inhibition caused by glycerol. Stevenson et al. [128] tested various procedures for their ability to retain the initial enzymatic activity by addition of silica gel to the reaction system, which being a hydrophilic substance, partially removed glycerol from the lipase environment. Use of n-heptane amended with acetone as a reaction mixture or use of a semi-continuous process consisting of a transesterification reaction and rinsing of the catalyst with a rinsing solution amended with water, which removes glycerol and also restored the thermodynamic water activity. Du et al. [119] observed that washing immobilized lipase after transesterification with isopropyl alcohol restored its activity as glycerol was removed from the carrier. Hydrophilic tert-butanol was used as an organic solvent in the transesterification of rape seed oil using *C. antarctica* lipase and *Tidestomia lanuginosa* lipase, which not only helped in the solubilization of methanol but also solubilised glycerol [125]. Therefore, the coating of the immobilized lipase by glycerol was controlled. Chen and Wu [107] proposed the use of tert-butanol or 2-propanol for washing immobilized *C. antarctica* lipase. Belati-Bako et al. [129] proposed the use of a membrane bioreactor using a suitable dialysis membrane. The reaction takes place in the primary side of the module and the module, and the glycerol produced during the reaction passes through the membrane and is accumulated in the secondary (aqueous phase). Another novel approach in countering glycerol inhibition was proposed by Xiu et al. [130] where in an integrated bioprocess combining biodiesel production using lipase and the conversion of glycerol 1,3-propane diol using bacteria *Klebsiella pneumoniae* in an hollow fiber membrane was carried out.

4. Production of biodiesel from microalgae

4.1. Algae

Algae can be directly converted into energy [131]. Algal Biomass grows in aquatic conditions and uses light and carbon dioxide for their normal metabolism and survival. There are two types of algae: Microalgae and Macro algae. Macro algae are multicellular and large usually grown in ponds and oceans characterized by varied growth patterns. Microalgae are diverse group of species, prokaryotic and eukaryotic, photosynthetic microorganisms. Examples of prokaryotic microalgae are cyanobacteria (*Cyanophyceae*) and eukaryotic micro algae are green algae (*Chlorophyta*) and Diatoms (*Bacillariophyta*) [132,133]. Microalgae are tiny, unicellular and multicellular organisms found growing in suspensions [134]. Microalgae are found to be rich in oil (Triacyl Glycerols) which highlights them as a prime base for biodiesel production. The growth of Microalgae is very quick and some species have been reported to have their doubling time in 3.5 h [8]. Algal harvesting consists of biomass recovery from the culture medium that may contribute to 20–30% of the total biomass production cost [135]. Usually microalgae have oil content ranging from 30 to 70% of dry weight depending on the species of consideration. This clearly portrays that algae can replace coal and oil producing crops in the near future. Though the global fuel demand intensifies exponentially, biodiesel from oil producing crops cannot satisfy existing demand realistically and algal biofuels draws the attention of satisfying the same [138,139].

4.2. Algal diversity and global collection centers

It is estimated that 50,000 species of micro algae exists, but only 30,000 have been studied and analyzed and 15 were used commonly [139]. There are many microalgae collection centers in the world that provides mother culture for further use. Fresh water microalgae collection of Coimbra (Portugal) contains 4000 strains and 1000 species. Collection of Goettingen University has about 2213 strains and 1273 species. University of Texas Algal Culture

Collection was founded in 1953 and has about 2300 different species of microalgae. National Institute For Environmental Studies Collection (NIES), in Japan holds a collection of about 2150 strains and 700 species of different algae. The CSIRO Collection of Living Microalgae (CCLM) in Australia holds about 800 strains from Australian waters [134].

4.3. Biodiesel from microalgae

Microalgae require organic carbons for their normal metabolism and are categorized under autotrophs and heterotrophs. Photoautotrophic algal population finds the majority among algal species and basically requires light and CO₂ for their growth. To satisfy the capitalization factors and justify algal biomass for biofuel source, autotrophic algal biomass helps in upholding the focus and advances the economics of algal biofuel. Table 6 lists out the oil producing potential of few open pond algal species. Although microalgae can utilize light efficiently, the phototrophic growth of algae is too slow when compared with heterotrophic algae. There are factors like photo-inhibition, where algae is exposed to excessive light in sunny days, light limitations due to high cell density in large scale culture are the various factors affecting the performance of phototrophic algae. Hence, heterotrophic algal cultivation in conventional fermenters is suggested [142]. Heterotrophic cultivation of algae offers several advantages such as elimination of light requirements, low cost for harvesting, good and controlled cultivation process and higher cell density [143]. Cell growth can be influenced by medium supplied, and controlled environmental factors, where heterotrophic algal cultivation superiors to phototrophic algal cultivation.

4.4. Microalgae cultivation

Microalgal cultivation with suitable mediums was discussed and potential oil producing feedstocks are examined in lab scale [144,145]. Optimization parameters such as nitrogen, phosphorous concentration and outdoor parameters such as photoperiods can make a significant change in lipid productivity [144]. Lifecycle analysis by Jorquera et al. [146], Yang et al. [147] and Campbell et al. [148] enables the screening of suitable microalgal strains and the selectivity based on various parameters. Economical production of microalgae can be done in two common methods as follows.

4.4.1. Open ponds (or) raceways

For the production of phototrophic algae, open ponds are designed in raceway configuration usually 0.3 m deep and a paddle wheel circulates and mixes the algal cells with nutrients (Fig. 4). This system is continuous where fresh feed is added in front of the paddlewheel and the algal broth is collected behind the wheel after it has circulated the whole loop. For some marine type microalgae, seawater or high salinity water can be used. Open pond limits the biomass productivity as they do not allow microalgae to use CO₂ efficiently [8]. In addition, optimal culture conditions are difficult to maintain in open ponds and biomass recovering methods remains expensive [149]. About 25% of biomass produced during day light may be lost during night due to respiration factors. The extent of this loss depends on the light level and growth temperature at night. Besides certain advantages, still open ponds are the efficient ways to scale up the production of algal biomass in large quantities [149,150].

4.4.2. Tubular photobioreactors

It has been reported that large quantities of microalgal biomass can be successfully produced by tubular photobioreactors [149].

Photobioreactors permit the growth of single species of microalgae for long time durations (Fig. 5). Enclosed photobioreactors have been employed to overcome the problem of contamination and evaporation [149]. This system is made of transparent tubes and generally placed outdoors for natural light illumination. These cultivation vessels have good surface area to volume ratio to increase productivity. Tubes are generally less than 10 cm in diameter to maximize sunlight penetration. A continuous algal culture is made possible by circulating the medium broth through a pump to the tubes where it gets exposed to light and then sent back to reservoir. A portion of algae is harvested after it passes through the tubes. Spirally coiled tubes form the basic structure of helical shaped bioreactors [149]. The biomass productivity of photobioreactors can average 13 times more than that of a traditional raceway pond. Biomass harvesting in this method remains easy because of the biomass concentration which is 30 times the concentration of biomass obtained via raceway method.

4.5. Triglyceride synthesis

The ultimate aim of cultivation is lipid production which is the main substrate for biodiesel production. Like other higher plants and animals, algal cell also produces triglycerides to store substance and energy [151]. Generally, acetyl-coA and L-α-phosphoglycerol are the primary compounds in triglyceride production. Acetyl-coA and Phosphodihydroxyacetone, precursor of L-α-phosphoglycerol are obtained as a result of glycolysis process. Acetyl-coA reacts with the hydroxyl group of L-α-phosphoglycerol to give lysophosphatidic acid. This acid again combines with acyl-coA to form phosphatidic acid. Glycerol phosphate acyl-transferase catalyze the above two reactions. Further, lysophosphatidic acid is hydrolyzed by phosphatidate phosphatase to form diglyceride. Another acetyl-coA is combined with diglyceride to give triglyceride and this is catalyzed by glyceryl diester transacylase (Fig. 6).

4.6. Extraction and analysis of oil and biomass

Extraction of oil from algal biomass requires the need of cell disruption techniques. Mechanical cell disruption technique involves the use of press configurations such as crushers called as oil press. Crush configurations varies with algal species as the cell physiology changes. Cavitation caused by collapsing bubbles in ultrasonicator creates shock waves that can also be employed to disrupt the cell wall. Mechanical crushing assisted by chemical extraction methods have been reported best in performance. Petroleum ether suits best for solvent criteria in case of chemical technique of extraction where extraction efficiency exceeds 90% realistically. Typical characteristics of fossil oil and bio-oil from microalgae are studied [136]. For primary analysis of fatty acid profile of microalgal oil, approximately 50–100 mg (B₁) of dried algae can be lyophilized using a vacuum freeze dryer followed by soxhlet extraction with chloroform:methanol (2:1, v/v) as solvent. The weight of the supernatant is calculated (B₂) and then blow dried in a fume cupboard. The supernatant was dried to a constant weight at 105°C (B₃). The lipid content (C, %) and biomass productivity (P, mg L⁻¹ d⁻¹) were calculated using the following formulas [144]:

$$C (\%) = \frac{B_1 - B_2}{B_1} \times 100$$

where

B₁, B₂, B₃ – weight in grams.

$$P (\text{mg L}^{-1} \text{ d}^{-1}) = \frac{DW_2 - DW_1}{T_2 - T_1}$$

where

DW_1 , DW_2 are the dry weight (g L^{-1}) of the algal sample at inoculation and harvesting respectively. T_1 and T_2 represent the inoculation and harvesting time respectively.

4.7. Significance of algal biofuel

Microalgae are a diverse group of prokaryotic and eukaryotic microorganisms that grows rapidly due to their simple structure. Micro algal biodiesel production is potentially sustainable and in the nearest future it will be made possible to produce adequate amount of fuel to meet the fast growing fuel demands. Advancement in photobioreactor design, micro algal biomass harvesting and downstream processing techniques increases the efficiency of algal biofuel production and thereby algal fuels are likely expected to replace the conventional sources of biodiesel production. Properties of conventional diesel, microalgae derived biodiesel and ASTM biodiesel standards have been compared and found to be satisfactory [152]. Properties of algal fuels are given (Table 7 and 8). Though there are various extraction techniques for oil from algae, solvent extraction is efficient and suits industrial scale extraction [153].

Combining microalgae farming and the biofuel production using bio-refinery strategy is expected to significantly enhance the overall cost effectiveness of algal biofuels. Many researches have been undertaken and some are found to be in progress in making the algal biofuels significant in the biodiesel catalog with main targets such as, increasing oil content of existing strains or selecting new strains with high oil content, increasing the growth rate of algae and developing robust algal-growing systems in either open-air or enclosed environments. Developing co-products other than oil like usage of residual biomass for development of economically valuable products, using algae in bioremediation and developing an efficient oil-extraction method will make this concept of algal biodiesel a sustainable one.

5. Conclusion

The problem of oil crisis is the hotly debated topic worldwide since the mid 1970s which resulted in the search for new sustainable oil source. Though many plants and microbes are known to produce oil, most of them are not able to produce oil sustainably. Trends in microalgae biotechnology have contributed hopes for oil revolution in the field of alternate fuels, where as some of the algal species with low lipid content and low yield of biomass are the stoppage for industrial production. Though micro algal biomass has much significance both economically and environmentally, macroalgae is found to attract the researches because of its ease-to-grow and eco-friendly nature. Hence macroalgae biodiesel stocks may become a very attractive oil source as the latest explorations about their oil content turns confidence on investment in the area of macroalgae cultivation. However acid/alkali catalyzed process has great reaction rates, it remains undesired because of non-recoverability. Vegetable oil is the only sustainable oil feedstock as of today's research which is much compatible with two step acid/alkali catalysis or enzymatic catalysis for biodiesel production. Algal research such as growth optimization for high yield of biomass and oil, advancements in photo bioreactor engineering, good downstream processing practices coupled with intellectual selection of transesterification methods, innovation in technical aspects such as co-transesterification of waste cooking oil and algal oil may roll to bring progress in algal biodiesel production and can immediately effect oil sustainability as possible future improvements for biofuel production.

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